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PRINCIPAL INVESTIGATOR: Feng Wang-Johanning, M.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

E-Mail: fengj@uab.edu

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The human genome contains about 1% endogenous retroviral sequences. Most human endogenous retroviruses (HERVs) are defective because of multiple termination codons. Of the many HERV families, only K appears to have all structural features necessary for viral replication. In our previous studies, HERV-K transcripts with coding potential for the envelope region were expressed frequently in human breast cancer cell lines and tissues (45%), but not in normal breast cells or tissues. In the current project, HERV-K molecules that serve as novel tumor targets for the detection, diagnosis, and immunotherapy of human breast cancer will be evaluated. It is very critical to determine whether or not HERV-K protein is expressed in breast cancer. We therefore synthesized HERV-K *env* surface protein. The purified proteins were used to immunize rabbits to produce polyclonal antibody, and mice to produce monoclonal antibody. The expression of HERV-K *env* surface protein in human breast cancer tissues was evaluated by immunohistochemistry using anti-HERV-K antibodies. We detected protein expression in breast cancer tumor epithelial cells, but not in uninvolved epithelial cells from the same cancer patients, thus providing evidence that HERV-K *env* gene is not only transcriptionally active in breast cancer, but also has translational activity in breast cancer.

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SUBJECT: Annual Report for Award Number DAMD17-00-1-0123
TITLE: HERV-K 10 as a target of immunotherapy for breast cancer
P.I. Feng Wang-Johanning, M.D.

INTRODUCTION:

Human endogenous retroviruses (HERVs) are stably inherited sequences thought to have entered the germline of their host more than a million years ago (1, 2). These elements are widely dispersed throughout the genome and are estimated to comprise more than one percent of the entire human genome. Humans have a large number of endogenous retroviruses and retroviruslike elements in their genome, and most HERVs described so far have become inactive and replication-defective in humans during evolution. As noted above, the discovery of HERV mRNA, HERV proteins, and even HERV particles associated with human diseases, especially cancers, has increased in recent years. Although endogenous retroviruses are considered to have the potential for causing disease in animals and humans, the pathogenic potential of nondefective endogenous retroviruses has so far only been demonstrated in mice, in which they may induce tumors and immunological disorders. At present little is known about the pathological and physiological importance of endogenous retroviruses in human diseases.

HERV-K: The type K family (HERV-K) is present in an estimated 30 to 50 copies per human genome and includes some elements with long open reading frames (3) (4). This family of HERVs was originally identified by its homology to the mouse mammary tumor virus (MMTV), and contains members which are transcriptionally active in several human cancer tissues (1, 5, 6) as well as tumor cell lines, most notably the human breast cancer cell line T47D (7, 8). HERV-K genomic sequences were first detected in 1986, and HERV-K retrovirus-like particles have been described which encode for viral particles in the teratocarcinoma cell line GH (5, 6), and the breast cancer cell line T47D (9). HERV-K 10, a full-length proviral clone, has been sequenced and found to be defective (10). Several other reports suggest the existence of at least ten different sequence families in the human genome with varying degrees of homology to HERV-K10 and mouse mammary tumor virus (MMTV) *pol* genes, as well as to each other (11-13). It has been suggested that there are between 30 to 50 copies of HERV-K genomic DNA within the human genome; more recently this number has been increased to 170 (14). The HERV-K family of endogenous retroviruses is believed to be unique in several regards. Of the many HERV families, only K appears to have the full complement of open reading frames typical of replication competent mammalian retroviruses (LTR-gag-pro/pol-env-LTR) (10) (3).

In teratocarcinoma cell lines, HERV-K *gag* genes are expressed, leading to the production of viral core proteins and virus particles (6). HERV-K is expressed in a complex splicing pattern in testicular tumors and derived cell lines as well as placenta. The HERV-K genome is also spliced into subgenomic transcripts in the human breast cancer cell line T47D (8). The K family contains an additional ORF termed cORF (central), whose translated product is biologically comparable to the human immunodeficiency virus type-1 (HIV-1) Rev protein, critical for nuclear export of unspliced mRNA (6, 15-18).

In addition, an antibody response to the *gag* and *env* proteins of HERV-K has been identified in patients with seminoma, suggesting synthesis of these proteins *in vivo* (19), whereas healthy individuals and patients with inflammatory diseases very rarely develop such antibodies. The antibody titers of the patients showed a decrease with time after the patients received antitumor treatment or tumor removal (20). All these findings collectively indicate that HERV-K proteins constitute markers specific for germ cell tumors, but leave open the possibility that individual proteins exert functions that actively contribute to tumorigenesis.

In the past year, we discovered HERV-K *env* RNA expression in most of the human breast cancer cell lines and

biopsies analyzed (45% positive), but not in normal breast samples (21, see Appendix). More recently, studies in our laboratory have revealed the presence of multiple spliced transcripts of HERV-K in breast cancer samples that encoded open reading frames. Our data provide a strong indication that HERV-K transcripts with coding potential for the envelope region are expressed frequently in human breast cancer. These findings suggest important roles for HERVs in human physiology and pathophysiology, including carcinogenesis, and raise the possibility of HERV-K involvement in human breast.

BODY:

Hypothesis: Our hypothesis is that human endogenous retrovirus K (HERV-K) *env* protein might be an appropriate target for immunotherapy of breast cancer.

To test our hypothesis, we propose the following three Specific Aims:

Specific Aim 1. Detect or characterize HERV-K *env* protein expression in malignant, benign, or normal breast tissues:
Task1. To purify large amounts of HERV-K10 *env* gene recombinant proteins (months 1-6)
Task 2. To produce polyclonal and monoclonal antibodies against HERV-K10 *env* protein (months 7-12)
Task 3. To identify HERV-K10 *env* protein expression using anti-HERV-K10 antibodies (months 13-18)

Specific Aim 2. Analyze breast cancer patient sera (and appropriate controls) for antibody to HERVs
Task 1. To identify anti-HERV-K10 *env* antibody in patients with breast cancer or other disorders as well as normal breast tissues by ELISA and Western blot using anti-HERV antibodies (months 19-24)
Task 2. To identify HERV-K10 *env* fusion proteins in a prokaryotic system using the antisera from breast cancer patients (months 19-24)

Specific Aim 3. Determine ability to induce human lymphocytes to become immune to HERV proteins using HERV-K 10 transfected autologous dendritic cells for *in vitro* sensitization:
Task 1. Generate the dendritic cells from peripheral blood mononuclear cells (PBMCs) (months 25-36)
Task 2. In vitro transcription of HERV-K 10 mRNA (months 25-36)
Task 3. Transfection of DCs with RNA (months 25-36)
Task 4. Induce and assess HERV-K10 antigen-specific T cell activity (months 27-36)

KEY RESEARCH ACCOMPLISHEMENTS:

Synthesis of HERV-K *env* fusion protein: First, we constructed an HERV-K *env* fusion protein in a GST (prokaryotic) expression system. HERV-K *env* cDNAs with an open reading frame derived from a breast cancer mRNA were cloned into a pGEX 4T-1 vector. Plasmids with correct *env* gene sized were induced by IPTG to produce fusion proteins and proteins were subsequently purified by affinity chromatography using GS 4B. Several clones including K10G17 produced full-length fusion proteins. K10G17 was further confirmed to produce HERV-K *env* surface protein by sequence analysis.

Production of monoclonal and polyclonal antibodies: Large-scale preparations of K10G17 fusion proteins were produced and purified by affinity purification. The HERV-K *env* protein was separated from GST vector by cleavage with thrombin following another affinity purification. The purified HERV-K *env* proteins were used to immunize New Zealand White rabbits in order to produce polyclonal antisera, and to immunize Balb/c

mice to produce monoclonal antibodies. We successful produced the polyclonal anti-HERV-K *env* antibodies, but not monoclonal antibody.

Production of HERV-K *env* protein in a 6-His expression vector: Due to the large size of the GST protein itself (26 Kda), or for other reasons, we were not able to produce monoclonal antibody against HERV-K *env* protein. K10G17 insert was reamplifed and subcloned into a QIA expression vector, pQE30, which results in placement of the 6-His tag at the N-terminus of the HERV-K protein. K10G17Q18 was selected to produce HERV-K *env* surface protein with an expected size protein of 41 kilodaltons (Figure 1). The authenticity of K10G17Q18 was further confirmed by sequence analysis, and this protein was used to immunize mice for production of monoclonal antibodies. The sera from mice immunized with K10G17Q18 was used to test the specificity against HERV-K *env* protein by ELISA analysis (see Figure 2). The sera reacted only with K10G17Q18 (K10-HIS, in pQE30 vector) and K10G17 (K10-GST, in GST vector), but not with ERV3G4 (E3G4, another HERV *env* protein in a GST vector) and HERV-E4-1Q31 (A033/31, another HERV *env* protein in a pQE vector). The spleen cells obtained from this mouse have been fused and treated with drug in order to select clones to produce monoclonal antibodies specifically against HERV-K *env* protein.

Detection of HERV-K *env* protein expression in human breast cancer tissues: This entire grant proposal is based on the hypothesis that HERV-K *env* mRNA is not only actively transcribed in human breast cancer tissues, but is also translated to protein in these tissues. It is thus very critical to test for HERV-K protein expression in human breast cancer tissues. To date, expression of HERV-K *env* protein in breast cancer has not been reported. In this proposal, anti-HERV-K polyclonal antibodies were used successfully to detect HERV-K *env* protein expression in human breast cancer tissues. Characterization of HERV-K *env* translational activity was employed in serial breast tissue sections by immunohistochemistry using our polyclonal antibody. HERV-K *env* protein was detected only in tumor epithelial cells derived from ductal carcinoma *in situ* or invasive ductal carcinoma tissues, but not in adjacent uninvolved epithelial cells from the same patient tissue sample (Figure 3). Also, we did not detect the expression of HERV-K *env* protein in some normal breast tissues we tested. These results matched the results we observed by RNA *in situ* hybridization using HERV-K antisense probe (Figure 4). These results demonstrate that the HERV-K *env* gene is expressed in neoplastic breast tissue not only at the level of mRNA, but also at the protein level.

FIGURES:

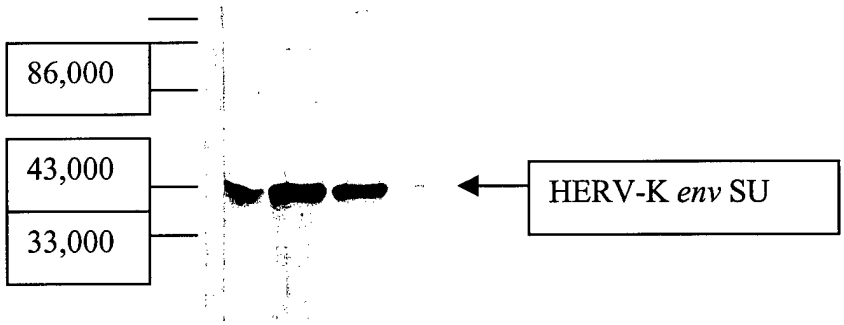

Figure 1. Production of HERV-K *env* SU *env* protein in a 6-His expression vector. Ten μg of HERV-K *env* SU fusion proteins purified by chromatography were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. Immunoblotting was performed with a mAb against RSG (anti-His mAb: 1:1,500 dilution, Qiagen), followed by treatment with anti mouse IgG AP secondary antibody (1:2,000 dilution, Sigma). HERV-K SU fusion protein clones, Cl1, (lane 2) and Cl2 (lane 3) produced the expected size fusion protein (41,000 daltons). Km is a Kaleidoscope prestained protein marker (Bio-Rad).

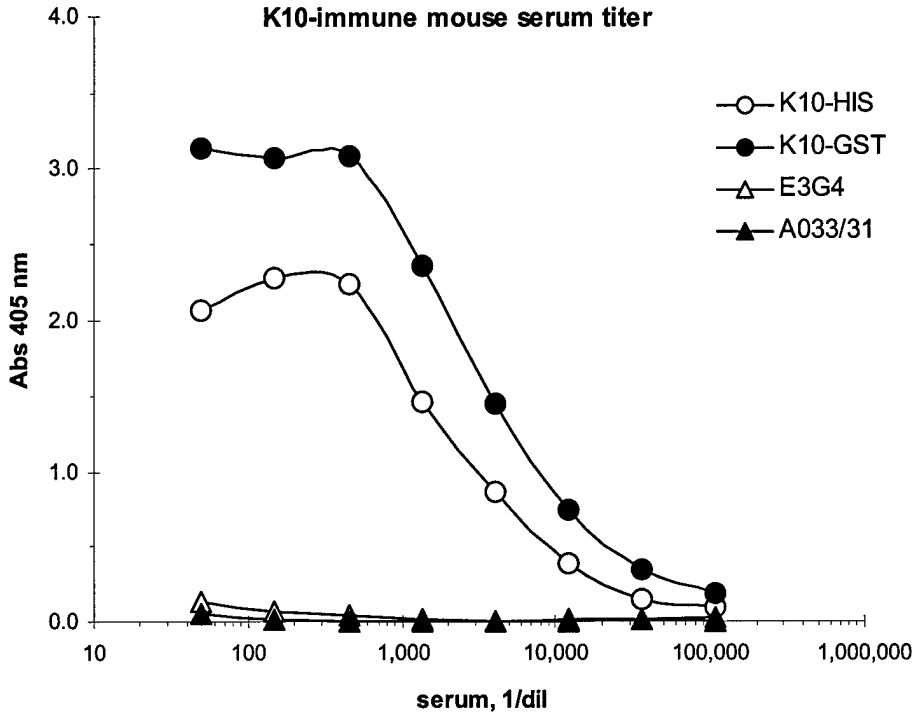

Figure 2. Binding affinity or specificities of anti-HERV-K sera: ELISA analysis of binding affinity or specificities of the antisera obtained from a mouse immunized with HERV-K *env* -6 His fusion protein. The ELISA plate was coated with various HERV fusion proteins (10 μg per ml, 100 μl per well) including K10G17Q18 (K10-His, HERV-K *env* SU cDNA in a 6-His vector), K10G17 (K10-GST, HERV-K *env* SU cDNA in a GST vector), ERV3G4 (E3G4, ERV3 *env* SU cDNA in a GST vector), and HERV-E4-1Q4 (A033/31, HERV-E4-1 *env* SU cDNA in a 6-His vector). Antisera were diluted from 50 to 109,350. The ELSIA plate was read at a wavelength of 405 nm.

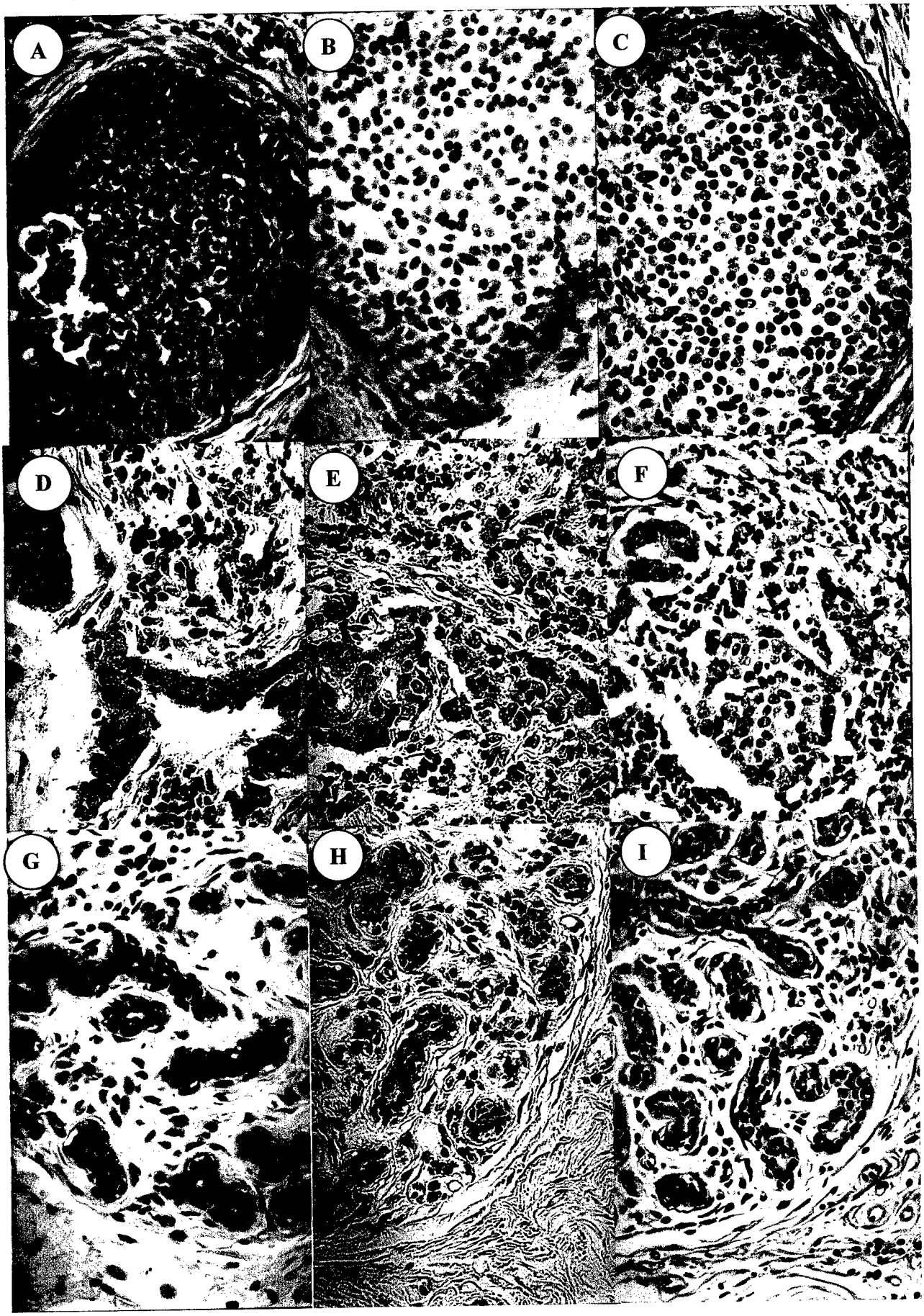
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Figure 3. Detection of HERV-K env protein expression in breast biopsies by immunohistochemistry. Serial breast tissue sections were assessed by immunohistochemstry using a polyclonal antibody specific against HERV-K env protein. Figures A to C; ductal carcinoma in situ. Figures D to F; invasive ductal carcinoma. Figures G to I; uninvolved breast tissues. Figures A, D, and G were incubated with anti-HERV-K polyclonal antibody. Figures B, E, and H were incubated with preimmune rabbit sera as a negative control. Figures C, F, and I were stained with hematoxylin and eosin. Phosphatase-stained sections were visualized (red) by light microscopy.

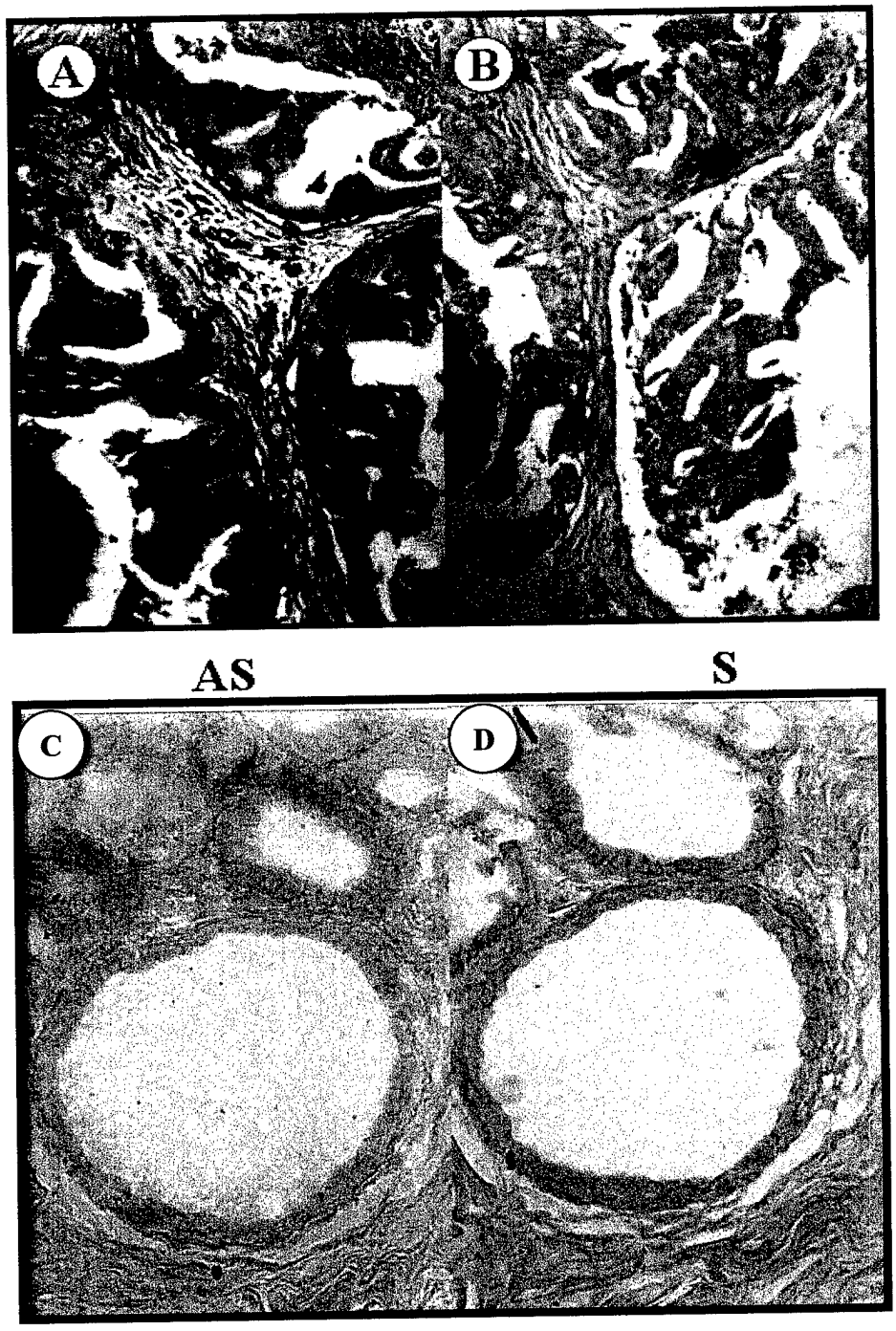

Figure 4. Detection of HERV-K transcripts in breast specimens by *in situ* hybridization. Characterization of HERV-K transcriptional activity in serial breast tissue sections by *in situ* hybridization using a digoxigenin-labeled HERV-K *env* RNA probe. Figure A and B; breast ductal carcinoma *in situ*. C and D, uninvolved breast tissues. Sections depicted in A and C were hybridized with HERV-K *env* RNA antisense probes (AS), while those depicted in B and D were hybridized with HERV-K RNA sense probes (S). Sections were visualized by light microscopy.

REPORTABLE OUTCOME:

None.

CONCLUSION:

In summary, we successful produced polyclonal antibody specific against HERV-K *env* surface protein and are in the process of producing monoclonal antibodies against this protein. It is very important that our data so far provide evidence that HERV-K *env* is not only transcriptionally active in breast cancer, but also has translational activity in breast cancer. Our results support the hypothesis that HERV-K *env* protein may be a novel molecular target for detection, diagnosis, and therapy of human breast cancer.

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Appendices:

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Advances in Brief

Expression of Human Endogenous Retrovirus K Envelope Transcripts in Human Breast Cancer[1]

Feng Wang-Johanning, Andra R. Frost, Gary L. Johanning, M. B. Khazaeli, Albert F. LoBuglio, Denise R. Shaw, and Theresa V. Strong[2]

The Comprehensive Cancer Center and Division of Hematology Oncology, Departments of Medicine [F. W-J., M. B. K., A. F. L., D. R. S., T. V. S.], Pathology [A. R. F.], and Nutrition Sciences [G. L. J.], University of Alabama at Birmingham, Birmingham, Alabama 35294-3300

Abstract

Purpose: **We investigated the expression of human endogenous retroviral (HERV) sequences in breast cancer.**

Experimental Design: **Reverse transcription-PCR (RT-PCR) was used to examine expression of the envelope (*env*) region of ERV3, HERV-E4-1, and HERV-K in breast cancer cell lines, human breast tumor samples, adjacent uninvolved breast tissues, nonmalignant breast tissues, and placenta. Expression of HERV transcripts was confirmed by Northern blot analysis and *in situ* hybridization (ISH). To evaluate coding potential, amplified HERV sequences were cloned into vectors for expression and sequence analysis.**

Results: **No expression of ERV3 or HERV-E4-1 RNA was detected in the analyzed breast samples. In contrast, HERV-K transcripts were detected in most breast cancer cell lines and many breast tumor tissues. Expression was detected in a small percentage of matched, uninvolved breast tissues and in placentas but not nonmalignant breast tissues. In HERV-K-positive breast cancer tissues, Northern blot analysis demonstrated full-length proviral and spliced *env* transcripts. ISH demonstrated expression of HERV-K transcripts in breast tumor cells but not in normal or uninvolved breast epithelial cells. Independently isolated clones of HERV-K *env* cDNA generated recombinant proteins of the expected size. Sequence analysis of *env* cDNA clones derived from four breast tumor samples revealed >97% identity with the type I HERV-K102, with no premature termination codons. Independent isolates from the same breast tumor sample showed nucleotide sequence differences, suggesting that multiple loci may be transcribed.**

Conclusions: **These data indicate that HERV-K transcripts with coding potential for the envelope region are expressed frequently in human breast cancer.**

Introduction

HERVs[3] are stably inherited sequences thought to have entered the germ line of their host more than a million years ago (1, 2). These elements are widely dispersed throughout the genome and are estimated to comprise >1% of the entire human genome. Most HERVs are defective because of multiple termination codons and deletions (3), but some appear to contain all structural features necessary for viral replication (4). HERVs are grouped into single- and multiple-copy families, usually classified according to the tRNA used for reverse transcription (2). The type K family (HERV-K) is present in an estimated 30–50 copies/human genome and includes some elements with long open reading frames (5, 6). This family of HERVs was originally identified by its homology to the mouse mammary tumor virus and contains members that are transcriptionally active in several human cancer tissues (1, 7, 8) as well as tumor cell lines, notably in the human breast cancer cell line T47D (9, 10). Two general types of HERV-K genomes exist, distinguished by the absence (type 1) or the presence (type 2) of 292 nucleotides at the boundary of the putative *pol* and *env* genes (4, 10).

Additional endogenous retroviral sequences that may be transcriptionally active in humans include ERV3 and HERV-E. ERV3 is a single-copy, full-length provirus that contains a nondefective *env* glycoprotein gene (11) and functional long terminal repeats. ERV3 *env* mRNAs are expressed in placenta and some tumor cell lines (12–15). HERV-E is a multiple-copy HERV family that contains long open reading frames in the *pol* and *env* regions of the provirus, indicative of the potential for expression (16). Expression of transmembrane env protein from the prototypic HERV-E, HERV-E4-1, has been reported in some cancer cell lines including colon carcinoma, germ cell tumors, and prostate adenocarcinoma (17).

The widespread distribution of multiple endogenous retroviral elements in mammalian genomes suggests that they may perform significant biological roles in the host and thus have been evolutionarily conserved. Several functions for HERVs have been proposed. Reverse transcription and integration of retroviral elements may contribute to the plasticity of the genome, accelerating the evolution of new genes (18) and altering the transcription of existing genes (19). HERV-encoded proteins

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[2] To whom requests for reprints should be addressed, at WTI 558, 1530 Third Avenue South, Birmingham, AL 35294-3300. Phone: (205) 975-9878; Fax: (205) 975-5650; E-mail: theresa.strong@ccc.uab.edu.

[3] The abbreviations use are: HERV, human endogenous retrovirus; RT-PCR, reverse transcription-PCR; env, envelope; HME, human mammary epithelial; ISH, *in situ* hybridization; GST, glutathione *S*-transferase; IDC, invasive ductal carcinoma.

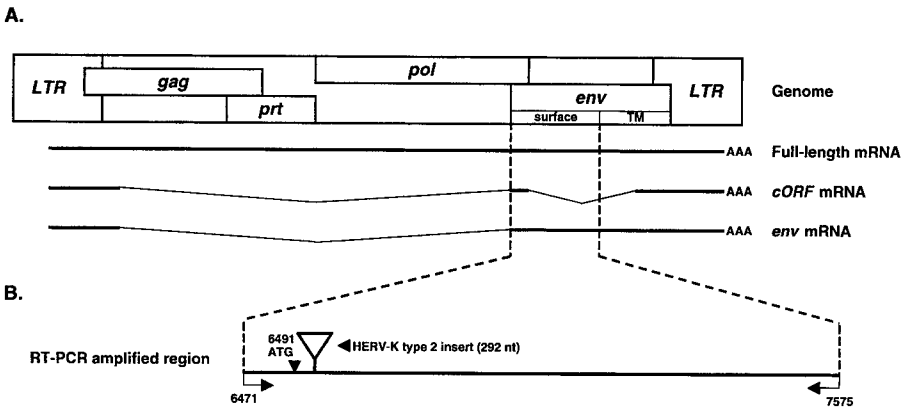

Fig. 1 HERV-K genome structure and RNA transcripts targeted by RT-PCR. *A*, generalized HERV-K genomic organization, showing only RNA transcripts that include *env* coding regions. *B*, diagrammatic representation of HERV-K *env* RT-PCR product targeted in the current study. Nucleotide numbering is according to the GenBank sequence accession no. AF164610.1 for HERV-K102 (35), a type I HERV. Note that only the putative surface env protein coding region was amplified. *TM*, putative transmembrane region of env.

may also have functions *in vivo*. In the placenta, the env protein of HERV-W has been shown to mediate the formation of syncytiotrophoblasts, an important step in placental morphogenesis (20). Expressed HERV proteins may also contribute to autoimmune disorders (21), although this remains controversial (22). A causal relationship between endogenous retroviruses and human cancer has been explored, including the recent demonstration of the transforming ability of an HERV-K central open reading frame gene (*cORF*; Ref. 23). In mice, endogenous retroviral genes are transcriptionally silent in normal tissues but expressed in several well-studied murine tumor models (24, 25). In some of these models, the expressed env protein acts as a tumor antigen capable of inducing both antibody and T-cell responses (26–29). In humans, tumors of germ cell origin have been reported to express HERV-K transcripts (30–32), and in seminoma patients, the HERV-K10 env protein is reported to be a tumor antigen eliciting host antibody responses (33).

As part of an ongoing search for tumor antigens important in breast cancer, we analyzed expression of the *env* region of ERV3, HERV-E4-1, and HERV-K in human breast cancer cell lines and tissues. Here we demonstrate that HERV-K transcripts are specifically and frequently expressed in human breast cancer, and that some of these transcripts contain open reading frames capable of producing env protein.

Materials and Methods

Cells and Tissues. Human breast cancer cell lines (BT-20, ZR-75-1, MCF-7, SKBr-3, MDA-MB-231, MDA-MB-453, BT-474, and T47D) were obtained from the American Type Culture Collection (Rockville, MD). Normal HME cells were obtained from Clonetics (San Diego, CA). Breast cell lines were cultured in the medium recommended by the American Type Culture Collection. The human teratocarcinoma cell lines Tera I and Tera II were kindly provided by Drs. Gail H. Vance and Virginia C. Thurston (Indiana University School of Medicine) and were cultured in α-MEM (Mediatech, Herndon, VA) with 10% fetal bovine serum (HyClone) and insulin (6 ng/ml; Sigma Chemical Co., St. Louis, MO). T47D cells were treated with 10 nM β-estradiol (Sigma Chemical Co.) and 100 nM progesterone (Sigma Chemical Co.) as described previously (34). Human breast tumor tissue, nonmalignant breast tissue from patients with other breast disorders (primarily fibroadenomas), normal human breast tissue from reduction mammoplasty, and placenta were provided by the Tissue Procurement Shared Facility of the Comprehensive Cancer Center at the University of Alabama at Birmingham, with Institution Review Board approval. Representative samples from breast tumor tissue and adjacent uninvolved tissue were isolated based on gross inspection. Tissue samples were snap-frozen and stored at −70°C until RNA isolation. For *in situ* hybridization, both snap-frozen and formalin-fixed, paraffin-embedded tissues were used. For Northern blot analysis, RNA from normal breast tissue (pooled from eight individuals) was purchased from Clontech Lab, Inc. (Palo Alto, CA), and RNA from breast cancer tissues (invasive ductal carcinoma) was purchased from BioChain Institute, Inc. (Hayward, CA).

RNA Preparation. Total RNA from cell lines and breast tissue samples was isolated using RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX) following the protocol provided by the manufacturer. RNA was treated with DNase (RNase-free; 1–2 units of DNase I/μg of DNA; Ambion, Austin, TX) at 37°C for 30 min to remove contaminating DNA, followed by heating at 75°C for 5 min to destroy residual DNase activity.

PCR Primers. Oligonucleotide primers derived from the *env* sequences encoding the putative env surface protein of ERV3 (nucleotides 786-2530; Ref. 11), HERV-E4–1 (nucleotides 6211–7559; Ref. 16), and HERV-K (nucleotides 6471–7575; Ref. 4) were used to amplify cDNA prepared from human tissues and cell lines (Fig. 1). Primer sequences were as follows: ERV3 sense, 5′-ACACTACGTGTCGGGGAACATCATG; ERV3 antisense, 5′-ACCAACCTCTGAAAAGGGAATCTGG; HERV-E4-1 sense, 5′-CTGGTCCACGCACGGCCGAAGCATG; HERV-E4-1 antisense, 5′-AAAAGGACGACTTAATAGAGCCAAT; HERV-K sense, 5′-AGAAAAGGGCCTCCACGGAGATG; HERV-K antisense, 5′-ACTGCAATTAAAGTAAAAATGAA. The HERV-K primers are 100% homologous to the following published HERV-K type 1 *env* sequences: K10 (GenBank accession no. M14123.1), K101 (GenBank accession no. AF16409.1), K102 (GenBank accession no. AF164610.1), K103 (GenBank accession no. AF164611.1), chromosome 5 (GenBank accession no. AC016577.4), and chromosome 19 (GenBank accession no. AC008996.5). These primers are expected to amplify both unspliced and spliced *env* transcripts. All HERV oligonucleotides were synthesized by Life Technologies,

Inc. (Grand Island, NY). Control primers to amplify human β-actin were from Stratagene (La Jolla, CA).

RT-PCR. Isolated RNA was incubated at 65°C for 10 min, followed by incubation on ice for 2 min prior to reverse transcription. Total RNA from each sample was reverse transcribed using cDNA synthesis beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) as per the manufacturer's directions. The reverse transcribed samples were amplified in a volume of 50 μl containing 3.3 μl of the first-strand cDNA synthesis mixture (corresponding to 1 μg of input RNA), 5 μl of 10× PCR buffer (Qiagen, Valencia, CA), 0.5 μl of Ampli-Taq DNA polymerase (2.5 units; Qiagen), and various sense and antisense oligonucleotide primer pairs at 50 pmol each. Each sample was analyzed in parallel with human β-actin primers. To assure that observations were not attributable to DNA contamination, all RNA samples were treated with DNase before cDNA synthesis. Additionally, 1 μg of RNA from each sample without reverse transcription was PCR amplified to control for genomic DNA contamination. PCR reactions were initially denatured at 94°C for 4 min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min). Amplified products were analyzed on a 1% agarose gel.

Northern Blot Analysis. Samples of total RNA (15 μg/lane) were electrophoresed at 5 V/cm on a 1.2% agarose-formaldehyde gel. After electrophoresis, gels were washed and transferred overnight onto a positively charged nylon membrane (Roche Molecular Biochemicals, Indianapolis, IN) using a Transblotter apparatus (Schleicher and Schuell, Keene, NH) and 10× SSPE buffer (1.5 M NaCl, 0.1 M NaH_2PO_4, and 20 mM EDTA, pH 7.4). Membranes were stained with methylene blue solution to visualize 18 S and 28 S bands.

The probe for Northern blot analysis was prepared using an *env* cDNA derived from patient 1 breast tumor RNA (Fig. 6, *Pt1, clone #2*), corresponding to the HERV-K102 sequence from nucleotides 6471–7575 (GenBank accession no. AF164610.1). The cDNA was labeled with [^{32}P]dATP using the random-primed labeling method. Labeled probe was hybridized overnight at 62°C with the membrane, using a high-efficiency hybridization buffer (Molecular Research Center, Cincinnati, OH). Membranes were washed three times at room temperature with prehybridization/wash solution (1× SSC, 1% SDS), followed by three washes at 65°C with the same solution, and exposure to autoradiography film at −70°C for 24 h.

***In Situ* Hybridization.** RNA probes were prepared from patient 1, HERV-K *env* clone #2 (Fig. 6). One μg of the linearized plasmid was used as template. The *in vitro* transcription and labeling of probe was performed at 37°C for 2 h with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals) using T7 and SP6 RNA polymerases to obtain run-off transcripts of the antisense (complementary to the mRNA) or sense (negative control) probes. Paraffin-embedded breast tissue specimens were cut into serial 5-μm sections, melted, deparaffinized in xylene, rehydrated in ethanol, and then fixed in 4% paraformaldehyde. Snap-frozen breast specimens were cut into 5-μm serial sections and fixed in 4% paraformaldehyde directly. After fixation, tissue sections were treated with proteinase K (20 μg/ml in 50 mM Tris-HCl, 5 mM EDTA) at 37°C for 15 min, washed with PBS, and incubated at room temperature in 0.1 M triethanolamine-HCl plus 0.25% acetic anhydride. One section from each group was pretreated with RNase A (Sigma Chemical Co.) before proteinase K treatment as a control. Hybridization of RNase-treated sections with antisense RNA probe verified that RNA and not genomic DNA was the target of the hybridization. Tissue sections were equilibrated in Quick-Hyb hybridization buffer (Stratagene, La Jolla, CA) for 30 min. Antisense or sense riboprobes were denatured and added with salmon sperm DNA (250 μg/ml; Stratagene) to the tissue sections and incubated at 68°C overnight. Sections were washed twice with 0.2× SSC, 0.1% SDS and then once with STE buffer (0.5 M NaCl, 1 μM EDTA, and 0.02 M Tris) at room temperature. After treating with RNase A (50 μg/ml in STE buffer) at 37°C for 45 min to minimize nonspecific binding, the sections were washed once with STE and once in 0.1× SSC plus 0.1% SDS at 68°C for 15 min. Detection of digoxigenin-labeled nucleic acids was by enzyme immunoassay with a digoxigenin nucleic acid detection kit (Roche Molecular Biochemicals), using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad, Hercules, CA), followed by development at room temperature for 1–2 h in the dark. Sections were then rinsed in deionized water and counterstained with methyl green (Zymed, South San Francisco, CA) for 15 min. Adjacent sections were hybridized with sense probe as a negative control or with H&E for histological evaluation.

Synthesis of HERV-K env Fusion Protein. For construction of GST-fusion protein expression constructs containing partial HERV-K *env* sequences, the following modified RT-PCR primers were used: sense 5′-CCGGAATTCGTAACACCAGTCACATGGATG (nucleotides 6494), antisense 5′-ATAGTTTAGCGGCCGCTCTTTTGGATCTATTTAAAACACC (amplifies nucleotides 6494–7552; GenBank accession no. M14123.1), with engineered recognition sequences for restriction enzymes (*Eco*RI and *Not*I, respectively) underlined. RT-PCR products from patient breast tumor samples were cloned into the pGEX 4T-1 GST gene fusion vector (Pharmacia). Plasmids with predicted *env* insert size were screened for protein production on a small scale using isopropyl-β-D-thiogalactopyranoside induction. GST fusion proteins were purified by affinity chromatography using glutathione Sepharose 4B (Pharmacia), subjected to 12% SDS-PAGE electrophoresis and transferred to nitrocellulose. Immunoblotting was performed with an anti-GST antibody (1:1000 dilution; Pharmacia). One of the clones that produced fusion proteins of the expected molecular weight was further characterized by nucleotide sequencing using vector-specific primers (Fig. 6, *clone #1* from *Pt1*).

Cloning and Sequencing of HERV-K RT-PCR Amplification Products. RT-PCR products resolved on agarose gels were purified using the QIAquick gel purification kit (Qiagen), subcloned into pCR-II vectors (Invitrogen, Carlsbad, CA), and sequenced using vector-specific reverse and forward primers plus internal HERV-K *env*-specific primers. Sequence analysis and alignment were carried out with DNAsis for Windows (Hitachi Software, San Francisco, CA) and the GenBank database.

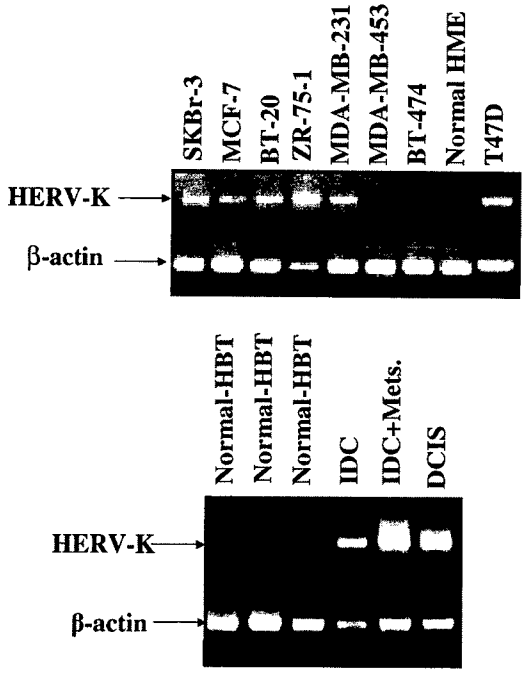

Fig. 2 Expression of HERV-K *env* RNA in breast cell lines and tissues. Ethidium bromide stained 1% agarose gel electrophoresis of RT-PCR products. Reverse transcribed RNA from breast cell lines and tissues was amplified by PCR (RT-PCR) using primers specific to the HERV-K envelope region and β-actin. *A*, RT-PCR amplification of RNA from SKBr-3, MCF-7, BT-20, ZR-75-1, MDA-MB-231, MDA-MB-453, BT-474, normal mammary epithelial cells (*HME*), and T47D. *B*, RT-PCR amplification of RNA from normal human breast tissues HBT (obtained from reduction mammoplasty) and breast cancer tissues including invasive ductal carcinoma (*IDC*, same as *Pt1* in Fig. 6), IDC with metastasis to lymph nodes (*IDC+Mets*, same as *Pt3* in Fig. 6), and DCIS (same as *Pt2* in Fig. 6).

Results

Detection of the *env* Region of HERV Transcripts in Breast Cancer Cell Lines and Tissues by RT-PCR. To investigate the expression of HERV elements in breast cancer cell lines, RT-PCR was performed using primers specific for *env* genes of ERV3, HERV-E4-1, and HERV-K. The HERV primer pairs for RT-PCR allowed detection of transcripts encoding the putative surface portion of each HERV env protein (Fig. 1). Expression of ERV3 and HERV-E4-1 RNA was not detected in breast tumor samples, although amplification of these products from placenta served as a positive control for PCR validation (data not shown). In contrast, HERV-K transcripts were detected in SKBr-3, MCF-7, BT20, ZR-75-1, MDA-MB-231 and T47D, but not in MDA-MB-453, BT-474, and normal HME cells (Fig. 2*A*). A single fragment of ~1100 bp, consistent with the size of a type 1 HERV-K *env* region, was detected. Because the primers were identical with several known type 1 HERV-K family members but contained 1–4 bp mismatches with known type 2 HERV-K env sequences, amplification of a single fragment was not unexpected. Expression of type 1 *env* transcripts in T47D cells has been reported previously (10).

These studies were next extended to surgical samples of breast carcinoma and nonmalignant breast tissues. Again, ERV-3 and HERV 4-1 transcripts were not detected in 43 breast tissue samples (20 tumor samples and 23 nonmalignant samples). In contrast, amplification products derived from the HERV-K *env* region were frequently detected (Fig. 2*B* and Table 1). To date, 130 breast tissue samples have been analyzed for expression of HERV-K transcripts by RT-PCR using the HERV *env* primers (Table 1). HERV-K *env* RNA was detected in 45% of breast cancer samples, in 18% of breast tissues derived from breast cancer patients but judged to be uninvolved by gross pathological dissection, as well as in 83% of placentas analyzed. In contrast, breast tissues derived from individuals not having breast cancer had no detectable HERV-K *env* expression ($n = 35$).

Table 1 Detection of HERV-K *env* gene expression in human tissue samples by RT-PCR

Sample description	No. of samples	HERV-K positive	
		No.	(%)
Breast carcinoma	55	25	(45)
Normal breast tissue from breast carcinoma samples	40	7	(18)
Normal breast tissue from patients without carcinoma[a]	35	0	(0)
Placenta	12	10	(83)

[a] Samples were from reduction mammoplasty ($n = 31$) and biopsies of fibrocystic lesions ($n = 4$).

HERV *env* Expression in Breast Cancer Cell Lines and Tissues Evaluated by Northern Blot Analysis. Northern blot analysis was used to further assess expression of HERV-K transcripts (Fig. 3). Using the *env* region as a probe (99% homologous to HERV-K102), positive radioactive species corresponding to the full-length proviral transcript (~8.3 kb) and the putative spliced *env* mRNA transcript (~3.0 kb) were detected in human breast cancer tissues but not in normal breast tissue (see Fig. 3). As a positive control, the full-length and *env* transcripts were also detected in the teratocarcinoma cell lines Tera I and Tera II (data not shown).

Expression of *env* Localized by RNA ISH. To demonstrate that HERV-K RNA was transcribed specifically in breast tumor cells, we performed *in situ* hybridization. Serial tissue sections were prepared and hybridized with an HERV-K *env* probe. HERV-K RNA was detected by ISH in 7 of 10 RT-PCR-positive breast tumor samples. This difference in the percentage of positive samples may reflect the lower sensitivity of ISH as compared with RT-PCR or may reflect differences in the specific area of each tissue sample used for analysis in the two independent assay types. In the positive tumor samples, the hybridization signal was detected specifically in the tumor cells and not in the surrounding normal cells. More importantly, when so-called "uninvolved" areas of breast cancer patient tissues were examined (*i.e.*, normal breast tissue from breast carcinoma samples), it became apparent that the positive signal detected in some of these samples by RT-PCR (Table 1) was likely attributable to the presence of previously undetected malignant cells (data not shown). Examples of ISH results are presented in Fig. 4, where a strong positive signal is detected in tumor epithelial cells of a tissue sample containing ductal car-

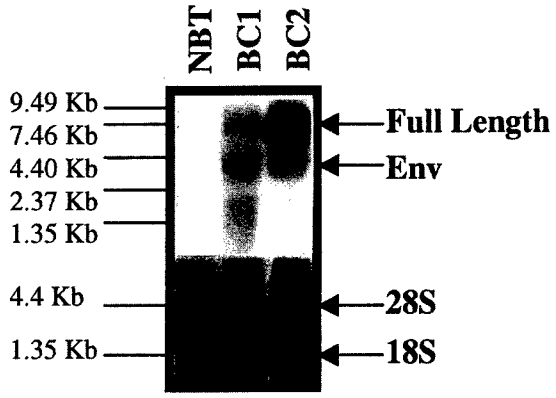

Fig. 3 Northern blot analysis of HERV-K *env* RNA expression in human breast tissues. Human tissues include normal human breast tissue (*NBT, Lane 1*) and two invasive ductal carcinoma samples (*BC1* and *BC2, Lanes 2* and *3*, respectively). Two transcripts are identified on the *right. Top band,* full proviral transcript. The bottom transcript presumably represents the spliced *env* mRNA. To verify the presence of RNA in all lanes, the blot was stained with methylene blue solution (*bottom panel*).

cinoma *in situ* (Fig. 4*A*), but no signal is detected in the matched, uninvolved epithelial cells of the same tissue sample (Fig. 4*C*). Matched tissue sections analyzed with the control sense probe were negative (Fig. 4, *B* and *D*). In addition, if the tissue was first treated with RNase, there was no signal using the antisense probe (data not shown). As expected from RT-PCR and Northern blot results, breast tissue from six individuals not having breast cancer had no detectable HERV-K RNA by ISH analyses. Thus, HERV-K transcripts were only detected in tumor cells by this assay.

Production of HERV-K env Protein from cDNAs Derived from Breast Cancer Tissues. As a test of the coding potential of the above expressed *env* sequences, HERV-K *env* cDNA amplified from selected breast cancer samples was cloned into a prokaryotic GST-fusion protein expression vector. The predicted recombinant fusion proteins would contain M_r ~26,000 of GST plus M_r ~41,000 daltons of HERV-K env surface protein. Six independent clones derived from a single breast tumor specimen (Fig. 6, Pt #1) were analyzed. Of these, four clones produced a fusion protein of the expected molecular size (M_r ~67,000), one produced a fusion protein of lower than predicted size, and one produced protein of the size of GST alone (partial results in Fig. 5). Similar results were obtained using clones derived from four additional breast tumor samples. HERV-K *env* cDNAs derived from the same tumor sample (Pt 1) were cloned into a eukaryotic expression vector for assay by coupled *in vitro* transcription-translation with [^{35}S]methionine. Radiolabeled protein of the size expected (M_r ~40,000) for the targeted HERV-K env product was detected in two additional clones (data not shown; sequence analysis in Fig. 6, *Pt1, clones #2* and *#3*). These results suggest that the amplified HERV-K *env* regions contained no premature stop codons and provide indirect evidence that such HERV-K env proteins can be stably expressed.

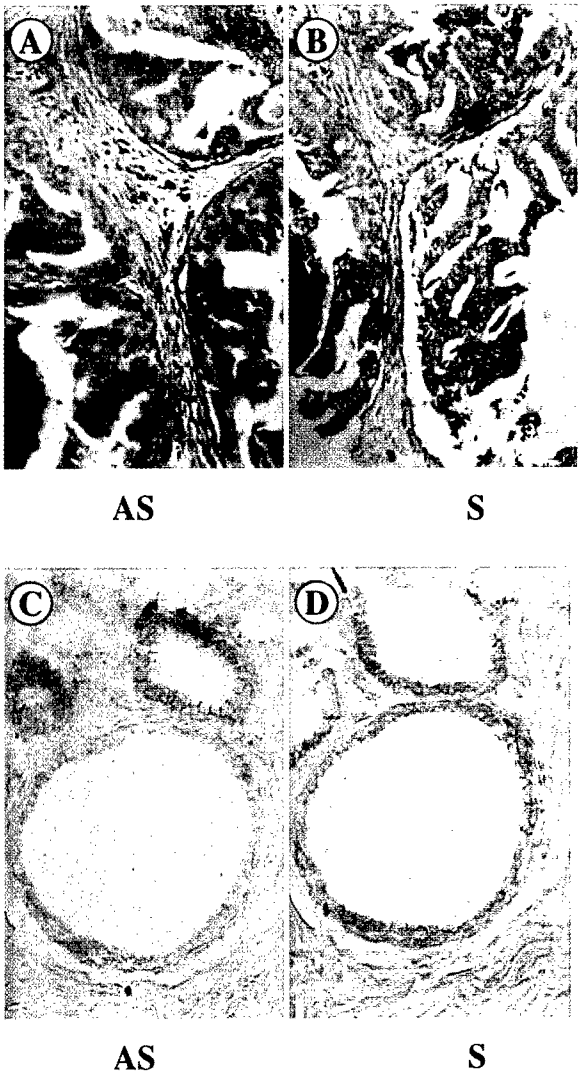

Fig. 4 Detection of HERV-K transcripts in breast specimens by *in situ* hybridization. Characterization of HERV-K transcriptional activity in serial breast tissue sections by *in situ* hybridization using a digoxigenin-labeled *env* RNA probe. *A* and *B*, breast ductal carcinoma *in situ*. *C* and *D*, uninvolved breast tissues. Sections depicted in *A* and *C* were hybridized with HERV-K *env* RNA antisense (*AS*) probes, whereas those depicted in *B* and *D* were hybridized with HERV-K RNA sense (*S*) probes. Sections were visualized by light microscopy.

Sequence Analysis of HERV-K Clones Derived from Breast Cancer Tissues. HERV-K *env* RT-PCR products derived from four individual breast cancer patient tissue samples were cloned, and eight independent isolates were completely sequenced (Fig. 6). All demonstrated >97% sequence homology to the previously reported HERV-K102 *env* (35), but none were identical to any published sequence. Sequence divergence from K102 was noted at 46 of 1035 nucleotides analyzed (4.4%). Of note, none of the isolated clones were identical to each other, even among clones derived from the same tumor sample, although specific nucleotides sequence divergence from K102 was in many cases shared among the eight clones. Specifically, nucleotide divergence was identical with at least one of

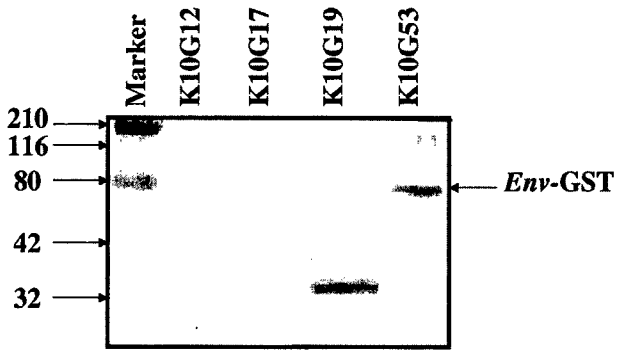

Fig. 5 Production of HERV-K env protein from a prokaryotic vector. Ten μg of HERV-GST fusion protein purified by glutathione Sepharose 4B chromatography were electrophoresed on a 12% SDS-PAGE gel and analyzed by Western blot. Shown are four of six independently isolated clones. *Lanes 1*, *2*, and *4* are full-length fusion proteins produced by clones K10G12, K10G17, and K10G53, respectively. Clone K10G19 in *Lane 3* did not produce a full-length protein. *Lane M*, Kaleidoscope prestained standards (Bio-Rad; molecular weight in thousands).

the other seven isolates for 13 of 46 nucleotide positions (28%). The remaining 33 nucleotide changes (72%) were unique among the eight cDNA clones. A few cDNA sequences showed highest homology to published HERV-K type 1 genes other than K102 (*e.g.*, *Pt1 clone #3* in Fig. 6 was most homologous to K101, GenBank accession # M14123), but in no case did the sequence homology exceed 99.5%.

Discussion

Endogenous retroviruses have several potential functional roles in their host. By analogy with mouse models of cancer, we hypothesized that proteins encoded by HERV *env* genes may act as tumor antigens. As a first test of this hypothesis, we used RT-PCR to examine expression of the *env* region of several candidate HERV genes in human breast cancer cell lines and surgical specimens. Whereas no breast tissue expression of ERV3 or HERV-E4-1 was found in our analyses, RT-PCR readily detected HERV-K transcripts in six of eight breast carcinoma cell lines and in 45% of the 55 analyzed breast tumor tissue samples. In contrast, a normal breast epithelial cell culture (HME) and multiple breast tissue samples from individuals not having breast cancer showed no detectable HERV-K *env* RNA. Northern blot analysis using an *env* probe derived from a breast tumor sample confirmed expression of HERV-K transcripts in breast cancer. Because a small percentage of samples judged to be nonmalignant by gross pathological inspection had detectable *env* RNA by the RT-PCR assay (18%), ISH was used to localize expression of HERV-K transcripts to individual cells by histological discrimination between tumor and adjacent uninvolved tissues. Such ISH studies indicated that small numbers of tumor cells present in some matched tissue samples previously identified as "uninvolved" based upon gross inspection could account for positive HERV-K *env* RT-PCR results for these samples (as in Table 1). Considered together, our RT-PCR and ISH results support the conclusion that expression of HERV-K transcripts is restricted to breast carcinoma and is undetectable in nonmalignant breast epithelial cells.

Expression of endogenous retroviral sequences has been implicated in a variety of human disease states (36). Of particular relevance to the current study, Yin *et al.* (37) previously described expression of HERV-K sequences in both normal and malignant breast tissue, using a classification system and assay targeting the *pol* genes of HERV-K subgroups HML 1–6. They reported that, on average, all HML groups were expressed at lower levels in breast tissues as compared with placenta, and furthermore, that HML *pol* genes were not more highly expressed in malignant as compared with nonmalignant breast tissues. Although the conclusions of Yin *et al.* (37) may seem in conflict with those from the current study, it should be noted that different retroviral gene products (*pol versus env*) and different HERV-K subgroups (HML *versus* K102) were targeted. It might be expected that gene-specific expression for individual HERV-K elements would correlate with overall transcriptional activity of HERV-K loci in any given cell type; however, this presumption is untested in the available literature and remains unexamined for samples used in the current study.

Whereas expression of various HERV mRNA species has been reported previously in different tumor tissues, HERV protein expression has been confirmed in only a minority of cases. Most HERV loci are thought to be defective, and certain HERV-K *env* sequences, including HERV-K10 and HERV-K107 (both type 1), are reported to contain a premature stop codon (TAA at nucleotide 6920) that should ablate env protein expression. The *env* cDNA clones sequenced in our study contained no stop codon at this position (codon was CAA instead of TAA for all eight clones), and no stop codons were observed over the entire *env* region analyzed in the presumed reading frame. Furthermore, our *in vitro* studies suggest that the HERV-K *env* cDNA clones from breast cancer tissue were capable of producing stable env protein of the predicted molecular size. Others have also reported expression of HERV-K env protein from cloned *env* cDNA after transfection into a mouse cell line (38). Thus, the potential for expression of HERV-K env proteins in human breast cancer is supported by independent data.

Sequencing of *env* cDNAs in the current study was performed in part to determine whether the transcripts were likely to have arisen from a single locus. Within the region of the HERV-K *env* gene sequence analyzed, the eight completely sequenced cDNAs amplified from breast cancer tissues showed >97% nucleotide homology to HERV-K102. Significant homology with HERV-K101, HERV-K10, and *env* sequences located on chromosomes 5 and 19 was also noted for individual clones. However, none of the eight sequenced clones were identical to each other or to any published *env* sequence. Although our sample size is small, it is interesting to note that many of the nucleotide changes were shared among clones isolated from either the same or an unrelated patient tumor specimen. Although it is possible that some of these nucleotide differences may have been generated during the amplification and cloning process, it is unlikely that this would account for all of the differences, particularly those specific changes observed in multiple clones. Also, inherited polymorphisms in HERV-K genes would not explain the cDNA sequence divergence among clones isolated from the same patient tumor sample. Random mutagenesis during the malignant transformation of breast tu-

tumor cDNA clones	6511	6530	6547	6549	6562	6567	6624	6632	6650	6654	6655	6657	6711	6787	6794	6874	6883	6929	6934	6994	7000	7031	7052
HERV-K102 genomic sequence, nt number	G	A	T	T	A	T	T	C	C	G	G	G	C	A	T	A	T	G	T	T	G	G	T
Pt1 #1	C													G			C						
Pt1 #2		G	C	A	C	C																	
Pt1 #3		G	C	A	C	C			T			C	T		C			A	C	C	A	A	C
Pt2 #1					C		C																
Pt2 #2		G	C		C	C		T		T								A	C		A		
Pt3 #1					C						A					G							
Pt4 #1					C																		
Pt4 #2		G	C	A	C	C																	

tumor cDNA clones	7077	7105	7112	7114	7119	7139	7244	7254	7262	7285	7286	7298	7300	7334	7352	7388	7392	7431	7483	7494	7495	7497	7513	% difference from HERV-K102 nt	aa
HERV-K102 genomic sequence, nt number	T	A	A	A	C	G	T	T	A	A	G	A	T	A	G	C	G	T	A	C	A	C	G	nt	aa
Pt1 #1					T																			0.4	0.6
Pt1 #2			G														C							0.7	1.4
Pt1 #3			G			A	C		G		C		G				C		G			T	A	2.4	4.6
Pt2 #1												G					C	C						0.5	1.2
Pt2 #2			G			A	C			G					T	T	C		G	T	G			1.8	3.5
Pt3 #1		T						C									C							0.6	0.6
Pt4 #1	G		G	G										G			C							0.6	1.2
Pt4 #2			G														C							0.7	1.4

Fig. 6 Analysis of nucleotide sequences of cloned RT-PCR amplifications of breast cancer patients. Sequence alignments were performed using DNAsis (Hitachi Software). HERV-K env clones derived by RT-PCR of patient (*Pt*) tumor tissue RNA were sequenced as described in the text. Consensus cDNA sequences were aligned with the HERV-K102 genomic sequence, GenBank accession no. AF164610 (35), using genomic sequence numbering spanning nucleotides 6494–7528. Only cDNA nucleotide differences from the HERV-K102 genomic sequence are shown, with each presumed amino acid (*aa*) difference denoted by a *shaded box*. No nucleotide deletions or insertions were observed in the analyzed cDNA clones with respect to the HERV-K102 genomic sequence. Pathological classifications of tumor samples used for cDNA cloning were as follows: *Pt1*, invasive ductal carcinoma; *Pt2*, ductal carcinoma *in situ*; *Pt3*, invasive ductal carcinoma with metastasis to lymph node; *Pt4*, invasive ductal carcinoma.

mor precursors would not seem to explain the identical nucleotide changes observed among clones derived from four individual patients. Because genomic sequences are not yet available for all of the estimated 30 or more copies of HERV-K in the human genome, it is difficult to estimate the potential impact of allelic variation on the current study. Considering the minimal estimated complexity of HERV proviral sequences within the human genome (39), clarification of the precise origins of the expressed *env* sequences studied here awaits more complete sequence data provided by the Human Genome Project.

Collectively, our results are in agreement with the hypothesis that multiple, related HERV-K gene loci may be transcriptionally activated in breast tumors. Such activation might occur because of similarities in HERV-K long terminal repeats, which might be activated in response to a transcriptional factor found specifically in the altered transcriptional milieu of malignant breast epithelial cells (40, 41). Alternatively, activation may be attributable to hypomethylation of genomic DNA. HERV-K sequences were reported to be hypomethylated in urothelial carcinomas (42), suggesting that a similar mechanism may function in breast carcinoma. Further investigations of the mechanisms regulating expression of the multiple individual HERV loci are needed to define those most likely to be active in human malignancy.

In summary, HERV-K *env* RNA was expressed in many of the breast cancer tissues and cell lines analyzed, with no expression detected in normal breast tissues, suggesting that *env* expression might be a tumor marker. These transcripts are likely to originate from multiple HERV-K loci, even within the same tumor. Similar analyses with expanded tumor samples will be needed to confirm these findings, to estimate the number of individual HERV-K loci transcribed in tumors, and to establish the potential prognostic or diagnostic value of *env* RNA and/or protein expression. Investigation of the mechanism resulting in HERV-K transcriptional activation in tumor cells has the potential to elucidate fundamental cell processes involved in malignant transformation.

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